Bioavailability and Biotransformation of Benzo(*a*)pyrene in an Isolated Perfused *In Situ* Catfish Intestinal Preparation

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In the aquatic environment, diet is an important route of exposure for the common contaminant and procarcinogen benzo(*a*)pyrene (BaP). Dietary organisms vary in their BaP content and in contaminated areas often contain other xenobiotics including cytochrome P4501A inducers. This study examined the effect of dose and previous dietary exposure to the inducer β-naphthoflavone (BNF) upon the intestinal metabolism of BaP and the systemic bioavailability of BaP-derived products in catfish. BaP was administered at 2 and 20 μM into *in situ*-isolated perfused intestines of control and BNF-pretreated catfish. The intestine formed an array of metabolites in all treatments including potentially hazardous metabolites such as BaP-7,8 and 9,10 dihydrodiols and 6-methyl-BaP. BNF treatment disproportionally increased the contribution of BaP-7,8 and 9,10 dihydrodiols relative to the contributions of other metabolites. A greater percentage of metabolites was evident as conjugates in 2 μM controls, whereas a greater percentage of unconjugated metabolites was evident for 20 μM controls and BNF treatments of both dosages. BNF pretreatment and the higher 20 μM BaP dosage resulted in greater bioavailability, with 2.6–5.5-fold and 3.0–6.3-fold increases in systemically available BaP products, respectively. Metabolites represented 10.2–23.1% of the increased bioavailability with BNF treatment, suggesting that mechanisms, in addition to induced metabolism, may be operative. These results indicate that intestinal bioavailability, level of biotransformation, and the metabolic profile of BaP-derived products entering the blood from the intestine may be altered by dose and dietary BNF pretreatment. *Key words*: activation, benzo(*a*)pyrene, bioavailability, biotransformation, catfish, elimination, *in situ* preparation, intestine. *Environ Health Perspect* 106:155–166 (1998). [Online 5 February 1998]
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Polynuclear aromatic hydrocarbons (PAHs) are frequently found as contaminants in impacted aquatic environments. These compounds have been associated with areas that demonstrate a high tumor prevalence in wild fish populations (*1–3*). The carcinogenicity of PAHs is a well-known consequence of their metabolic activation (*4*). Among the various PAHs, the model compound benzo(*a*)pyrene (BaP) has received the most attention in fish species, with a primary focus on hepatic metabolism and adduct formation (*5,6*). BaP has been shown experimentally to be carcinogenic to fish following both waterborne (*7*) and oral exposures (*8*).

The hydrophobicity and environmental partitioning (*9*) of BaP, as well as its accumulation in infaunal invertebrates (*10,11*), suggest that the diet may be an important route of exposure for aquatic species. Studies examining various levels in the aquatic food chain have demonstrated not only the presence of BaP in diet and tissues but the biotransformation of BaP by lower organisms and the subsequent food chain transfer of BaP molar equivalents (Meq) to higher organisms (*12–14*). (BaP molar equivalent is defined here as BaP and BaP-derived metabolites compositely quantified by radioactivity and placed on an equal molar basis using BaP's specific activity.)

Dietary BaP may also undergo biotransformation by the intestine of the consumer before entering the systemic circulation (*15*). Intestines of fish are capable of a wide variety of biotransformation reactions, some of which respond to dietary cytochrome P4501A (CYP1A) inducers (*16*). Dietary induction studies in both flounder (*17*) and catfish (*16*) indicate that under conditions of low inducer concentrations, select biotransformation activities in the intestine may equal or even exceed corresponding hepatic activities. Such induction effects may potentially alter the degree and pathway of metabolism. Similarly, *in vitro* BaP studies with flounder intestinal homogenates suggest that the biotransformation pathways and products formed in the intestine may be altered by the BaP dose (*17*). BaP may be activated or alternatively detoxified by metabolically favored pathways. These findings suggest that the balance between toxification and detoxification on first-pass metabolism through the intestine may depend on dose and previous exposure history.

Objectives of the present study were to examine the intestinal metabolism and bioavailability of BaP in context of the products formed under varying conditions of dose and previous exposure history. These goals were addressed using an *in situ* isolated perfused intestinal model (catfish). BaP dosages used were based on K_m determinations of relevant biotransformation enzymes in the intestine. Dietary pretreatment was accomplished using the CYP1A inducer β-naphthoflavone (BNF) at concentrations in the diet that would induce the intestine but not the liver.

Materials and Methods

Channel catfish (1,639 ± 300 g) were obtained from the Louisiana State University (LSU) Aquaculture Research Station, Baton Rouge, Louisiana. Fish for all treatment groups were maintained under flow through conditions in dechlorinated tap water (pH 8.10 ± 0.33; temperature 21.0 ± 1.1°C; total hardness 25.7 ± 7.2 mg $CaCO_3$/l; alkalinity 170.3 ± 18.2 mg/l) on a 12-hr light photoperiod. Fish were acclimatized at least 4 weeks to experimental conditions before use. All fish were held and treated in accordance with National Institutes of Heath guidelines.

Virgin Silvercup trout chow (Murray Elevators, Murray, UT) was fed to fish before experimental treatment. On initiation of experimental treatment, control fish were maintained on trout chow coated with corn oil (100 μl corn oil/100 g chow), whereas fish to be induced were fed 1 mg BNF/100 μl corn oil/100 g chow. Both dietary groups were maintained on appropriate experimental diets at 30 g feed/kg/day for 14 days before surgical manipulation. Fish were fasted 24 hr before surgery and *in situ* experimentation.

Intestinal microsomes were prepared from control and BNF-exposed catfish for use in *in vitro* studies of BaP positional

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metabolism and monooxygenase K_m and V_{max} determinations. Catfish were immobilized in ice water and sacrificed by severing the spinal cord (n = 4/treatment). Intestines were removed, rinsed thoroughly with ice-cold buffer 1 [0.25 M sucrose, 5 mM EDTA, 0.05M Tris-Cl, pH 7.4, 0.2 mM phenylmethyl sulfonyl fluoride (PMSF)] to remove contents, and cut open; mucosal cells were then removed by scraping with a scalpel. The mucosa was placed in 10 ml buffer 1 and the cells were sedimented at 2,000 × g, weighed, and homogenized in 4 vol buffer 1. Washed microsomal fractions were prepared by differential centrifugation using the procedure described by James and Little (*18*).

[^{3}H]-BaP for enzymatic assays and other standards was purchased from Chemsyn (a function of the NCI Chemical Carcinogen Repository; Bethesda MD). Before use, BaP was purified by neutral alumina column chromatography (Biorad, Melville, NY) with stepwise elution by hexane:toluene mixtures 98:2, 95:5, 90:10, 80:20, 50;50, and 0:100 (*19,20*). *In vitro* positional metabolism of purified [^{3}H]-BaP by intestinal microsomes was determined by radiochemical HPLC assay of all BaP metabolites (*18*). A fluorescence assay of phenolic BaP metabolites [aryl hydrocarbon hydroxylase (AHH)] was used to determine the K_m and BaP monooxygenase activities (*21*). K_m studies incorporated BaP concentrations ranging from 0.5 to 10 µM. Intestinal monooxygenase activities were also determined by assay of ethoxyresorufin-*O*-deethylase (EROD) activity (*22*). Ethoxyresorufin was prepared from ethyl iodide and resorufin as described previously (*23*) and purified by thin-layer chromatography (TLC) on silica gel plates. The formation of resorufin was monitored in a fluorescence spectrophotometer for 30 min. Cuvettes contained 0.1 M Hepes buffer, pH 7.6, 1% bovine serum albumin, 2 µM ethoxyresorufin, microsomes (1 mg), and 2 mM NADPH (added last) in a final volume of 3.0 ml.

In situ *protocols.* A total of 31 catfish were prepared for an isolated perfused intestinal segment. Two dosages of [^{3}H]-BaP (based on the K_m determined in the *in vitro* studies and environmentally relevant) in a semipurified micelle vehicle were examined in a variety of combinations with control or BNF-induced fish. The [^{3}H]-BaP, as the nonspecifically tritiated product (45–80 Ci/mmol; Amersham Corporation, Arlington Heights, IL), was introduced either into the intestinal lumen or into the blood perfusing the isolated segment. Six combinations of experimental parameters were examined: low BaP dose (2 µM)/non-induced/luminal (n = 5); low BaP dose (2 µM)/induced/luminal (n = 4); high BaP dose (20 µM)/noninduced/luminal (n = 4); high BaP dose (20 µM)/induced/luminal (n = 5); low BaP dose (2 µM)/noninduced/blood (n = 7); and low BaP dose (2 µM)/induced/blood (n = 6).

Surgical procedures. Catfish of either sex were anesthetized with buffered ($NaHCO_3^-$) tricane methane sulfonate (Argent Chemical Company, Redmond, WA) at induction and maintenance doses of 106 and 86 mg/l, respectively. On induction, individual catfish were administered 1.5 ml anticoagulant (75 mM sodium citrate:42 mM citric acid:136 mM dextrose) and transferred to an aquatic surgery table.

The intestine was exposed by a longitudinal ventral midline incision. A ventral loop of proximal intestine starting 10 cm caudad to the pylorus and extending aborally approximately 20 cm was used for the preparation (Fig. 1). The afferent (supply) vessel, a branch of the coeliacomesenteric artery, and the corresponding efferent (drainage) vessel perfusing the prospective intestinal segment as well as potential collateral vessels were identified and isolated, and suture (00 silk) was placed loosely around each. Once ligatures were in place, blood was drawn from the caudal vein into a syringe containing citrate anticoagulant. The syringe, with a total of 10 ml blood and 1 ml oxygen (as determined by preliminary blood gas studies), was placed on an orbital mixer that provided gentle agitation for the cells.

The isolated afferent vessel was cannulated using anticoagulant-filled PE-50 (polyethylene) tubing. Once secured in place, the line was cleared, attached to a syringe pump mounted on a rocking platform, and a blood flow rate of 0.1 ml/ min was established. After cannulation of the efferent vessel with PE-60 tubing, the ligatures loosely placed around collateral vessels were rapidly tied off. Efferent blood volume was measured to ensure that blood output equaled input so as to verify that the infusion and drainage were for the isolated segment. The preparation was transiently blanched with a small amount of anticoagulant in saline through the afferent cannula. Throughout blanching, the course was examined to ensure that the afferent cannulation supplied the intestinal segment before collection in the efferent cannula; to determine the efficiency or completeness of the intestinal segment perfusion; and to demarcate the length of intestine perfused in the soon-to-be isolated intestinal segment preparation. Immediately after blanching, the region of perfusion was noted and blood flow reestablished. Ligatures were then preplaced slightly in from the perfusion margin on the intestine to mark the isolated segment.

Dose preparation and introduction. The carrier vehicle for intraluminal [^{3}H]-BaP dosing was a semipurified micellar solution. The semipurified micellar solution was formulated fresh daily from 2.5 mM monooleoyl-rac-glycerol, 10 mM sodium taurocholate, 2.5 mM myristic acid (14:0), 2.5 mM palmitic acid (16:0), 2.5 mM stearic acid (18:0), 2.5 mM linoleic acid (18:2), and 0.9% saline. Micelles were formulated in 1 ml saline with the aid of sonication. [^{3}H]-BaP and nonradiolabeled BaP dosages were formulated at the experimentally determined

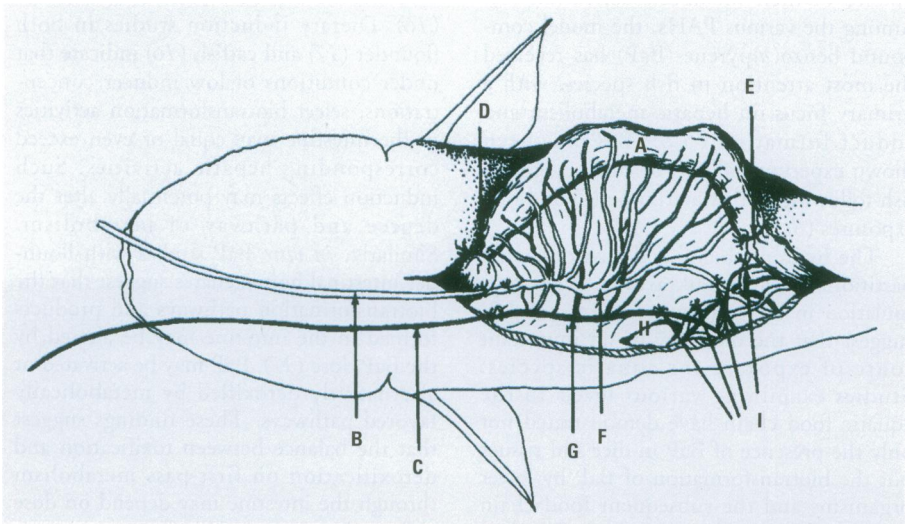

Figure 1. Diagrammatic representation of the catfish *in situ* isolated perfused intestine (ventral view). Letters indicate the following structures: A, isolated segment of intestine; B, efferent cannula; C, afferent cannula; D, proximal ligature and border of isolated intestine; E, distal ligature and border of isolated intestine; F, venous return; G, branch of coeliacomesenteric artery; H, spleen; I, ligatures on collateral vessels.

monooxygenase K_m (2 μM) and 10 K_m (20 μM). Purified [^{3}H]-BaP (370–640 μCi) in hexane and the balance of the dose as nonlabeled BaP in hexane were added to a conical tube containing saline (3 ml). N_2 gas was used to evaporate the solvent until only a surface film of hexane remained. The micellar mixture was introduced below the surface film. Initially the tube was gently mixed while the remaining hexane was evaporated, which allowed the bulk of the BaP to partition into the micelles. Once this was accomplished, more rigorous mixing dissolved the film until an essentially clear solution remained. Micelles were stable well beyond the experimental period (>24 hr).

At the time of dosing, the ligature around the distal extent of the perfused isolated intestinal segment was tied off. A small sample of micelle preinfusate was then collected for verification of BaP purity at time of analysis. The needle of the dosing syringe was inserted through the gut wall and the tip of the needle extended in the gut lumen for 2 cm beyond the proximal ligature. This ligature was then tightened over the needle allowing injection of the micellar solution and BaP dose into a nonperforated isolated intestinal segment. The injected dose was gently massaged throughout the entire segment, the timer started, and the room lights turned off to minimize photolysis of BaP.

In situ intestinal preparations in select experiments were dosed with [^{3}H]-BaP via the perfusion blood. [^{3}H]-BaP in an ethanol carrier was added directly to blood so that the BaP dose delivered during the timed perfusion was the same as that in the corresponding luminal study (2 μM). Once [^{3}H]-BaP was added to the blood, the carrier vehicle was evaporated and blood was throughly mixed and loaded into a syringe for perfusion. A micelle solution of identical composition (except for BaP) and volume as that used in the luminal studies was placed in the lumen of the intestinal segment as a collection medium.

Sample collection. Throughout the course of the 60-min perfusion, the intestine was moistened with saline (as needed), blood flow was checked every 10 min, and the isolated segment was gently massaged every 5 min. Whole blood collected compositely over the 60-min perfusion was sampled at each flow check to ensure proper sample representation. At the end of the perfusion, samples of liver, kidney, and anesthetic water were taken for radioactivity analysis to check the isolated preparation's perfusion integrity. (In intact isolated preparations, no radioactivity should be present in the samples.) The isolated ligated intestinal segment was removed intact and the length measured under standardized conditions. Postinfusate was collected by draining the intestinal segment, volumes were recorded, and aliquots taken for liquid scintillation counting and metabolite analysis. Once drained, the intestinal segment was cut open along the entire length, copiously washed with saline, and blotted dry. The mucosal surface was examined grossly for viability and then scraped with a glass slide. Mucosal tissue was mixed and aliquots were taken for liquid scintillation counting (approximately 100 mg) and metabolite analysis. Preinfusate, blood, liver, kidney, water, intestinal mucosa, and postinfusate were processed for liquid scintillation counting, as described below. Samples for metabolite determination (preinfusate, postinfusate, intestinal mucosa, and blood) were placed under nitrogen, wrapped in foil, and frozen at -20°C for up to 1 month until analysis.

Radioassay procedures. Tissue and postinfusate samples (approximately 100 mg) were digested in TS-1 (0.5 ml) tissue solubilizer (Research Products International, Mount Prospect, IL) for 24 hr at 50°C. After cooling, the samples were neutralized with 18 μl glacial acetic acid and counted in 4.5 ml scintillant (Ultima Gold, Packard, Downers Grove, IL). Radioisotope content was determined in water, preinfusate, and HPLC fractions following direct addition of the sample to scintillant. Radioactivity of all samples was corrected for counting efficiency (~50%) and background.

Analysis. Blood, mucosal cell, and postinfusate medium samples from the [^{3}H]-BaP-incubated *in situ* intestinal preparation were extracted by matrix solid phase dispersion, as described by James et al. (*20*). Briefly, samples of mucosal cells, 0.2–0.5 g, were added to 2 g prewashed, air-dried octadecylsilane-coated silica beads (C_{18}), 30–70 μm, (Alltech Associates, Deerfield, IL). The mixture was gently blended with a glass pestle to produce an evenly mixed, semidry material. This material, packed in a column, was eluted sequentially with 16 ml heptane and then with 8 ml each toluene, ethyl acetate, methanol, and water. Solvent eluents were collected in separate vials. After solvent elution, the packing material was extruded from the column, weighed, and mixed well. Five separate 0.1-g samples were then added to 18 ml scintillation cocktail and assayed for radioactivity. Aliquots of each solvent eluent were counted and the percentage of radioactivity in each fraction calculated. The heptane, toluene, ethyl acetate, and methanol fractions were dried separately under nitrogen, taken up in 0.25 ml 55% methanol, and filtered through a 0.45-μm centrifuge filter; the solutions were analyzed by HPLC. Selected aqueous fractions were hydrolyzed before analysis. Similar procedures were used for blood and postinfusate except that 0.2–1-ml samples of postinfusate and 3–6-ml samples of blood were used. Where sample size was greater than 0.5 ml, the amount of packing material mixed with the sample and the volume of eluent used were increased proportionally. The percentages of recovery, binding, and decomposition were determined by adding standards to mucosa, blood, and postinfusate and following the above procedure (Table 1).

Table 1. Percent recovery, binding, and decomposition of BaP and metabolite standards through the analytical procedure[a]

Compound	Tissue	Eluted	Bound	Breakdown
BaP	Blood	99.6	0.4	0.4
	Mucosa	99.8	0.2	2.0
3-OH-BaP	Blood	94.7	5.3	60 (to diones)
	Mucosa	98.1	1.9	50 (to diones)
9-OH-BaP	Blood	95.3	4.7	14.4
	Mucosa	98.2	1.8	22.1
BaP-7,8-diol	Blood	97.7	2.3	11.0
	Mucosa	98.3	1.7	7.9
BaP-9-sulfate	Blood	98.2	1.8	9.8
	Mucosa	96.0	4.0	4.5
BaP-9-glucuronide	Blood	84.4	15.6	2.8
	Mucosa	96.6	3.4	0.5

Abbreviations: BaP, benzo(*a*)pyrene; OH-BaP, hydroxy benzo(*a*)-pyrene; diol, dihydrodiol.

[a]Each radiolabeled standard was added to blood or mucosa at a concentration similar to those found in samples and immediately added to C_{18} packing material for analysis, as described in Methods.

The evaporated and reconstituted heptane samples were analyzed for BaP by isocratic reverse-phase HPLC with an octadecylsilane (C_{18}) column (4.5 × 50 mm precolumn with 4.5 × 250 mm column) and methanol as the mobile phase. The flow rate was 0.7 ml/min and the eluent was passed through UV-VIS (ultraviolet visable; Beckman model 332 set at 280 nm; Beckman Instruments, Palo Alto, CA), fluorescence (Shimadzu model RF 535 set at ex 375, em 435; Shimadzu Instruments, Columbia, MD), and radiochemical (Packard/Radiomatic Flo-one beta set for [^{3}H]; Packard Instrument Company, Meriden, CT) detectors. The evaporated and

reconstituted toluene, ethyl acetate, and methanol fractions were analyzed for BaP metabolites using a modification of the gradient system described by Salhab et al. (*24*). The column and detectors used were as for the isocratic separation, but the column was preequilibrated with starting mobile phase, 55% methanol:45% water, for at least 30 min before sample injection. After sample injection, the mobile phase was held at 55% methanol for 1 min. The amount of methanol was then increased by linear gradient to 86.5% MeOH over 30 min, held at 86.5% MeOH for 14 min, increased to 100% MeOH over 2 min, and held at 100% MeOH for 24 min. The column was then returned to the starting conditions. The retention times of BaP metabolite standards used in this system have been published elsewhere (*20*) except for BaP-3-, 7-, and 9-glucuronides, which eluted at 5.4, 5.8, and 5.6 min, respectively. Samples of dried methanol extracts and of the aqueous phase were incubated overnight at room temperature with β-glucuronidase or sulfatase, as described previously (*25*).

Statistical comparisons were performed with a 2-factor analysis of variance. To model the simultaneous effects of pretreatment and dose, statistical analyses of the experimental data were performed using the SAS (*26*) general linear models procedure. Pretreatment and dose level were modeled as separate main effects. A cross-products term was included to assess potential effect modification (interaction). In no instance did the interaction term have a significant impact on comparison of the main effects; therefore, the reported analyses are of the main effects only. Outcome variables were the concentration of metabolites in blood and mucosa, the total metabolites found in postinfusate, and the postinfusate fluid volume. Where appropriate, a Student's *t*-test was used for other comparisons.

Results

Low monooxygenase activity was evident in intestinal microsomes from control catfish. HPLC analysis of the BaP metabolites formed from microsomal incubations

Table 2. *In vitro* positional metabolism of BaP in intestinal mucosal microsomes from control catfish

Metabolite	Product (pmol) formed/min/mg protein
Triols and tetrols	0.43
9,10-Diol	0.26
7,8-Diol	0.23
Diones	0.85
7 and 9-OH-BaP	0.28
3-OH-BaP	1.28
Total activity	3.34

Abbreviations: BaP, benzo(*a*)pyrene; OH-BaP, hydroxy benzo(*a*)-pyrene; diol, dihydrodiol.

Table 3. Intestinal monooxygenase activities in catfish fed vehicle-treated chow or chow containing BNF, 10 mg/kg diet, for 2 weeks

	AHH		EROD
		V_{max}	
Treatment	K_m(μM)	pmol/min/mg	pmol/min/mg protein
Vehicle-treated chow	1.83 ± 1.06	12.5 ± 10.3	1.86 ± 0.92
BNF-treated chow	1.44 ± 0.46	70.2 ± 24.5*	11.5 ± 4.03*

Abbreviations: AHH, aryl hydrocarbon hydroxylase; BaP, benzo(*a*)pyrene; BNF, β-naphthoflavone; EROD, ethoxyresorufin *O*-deethylase activity.
*Significantly higher than control, $p<0.05$.

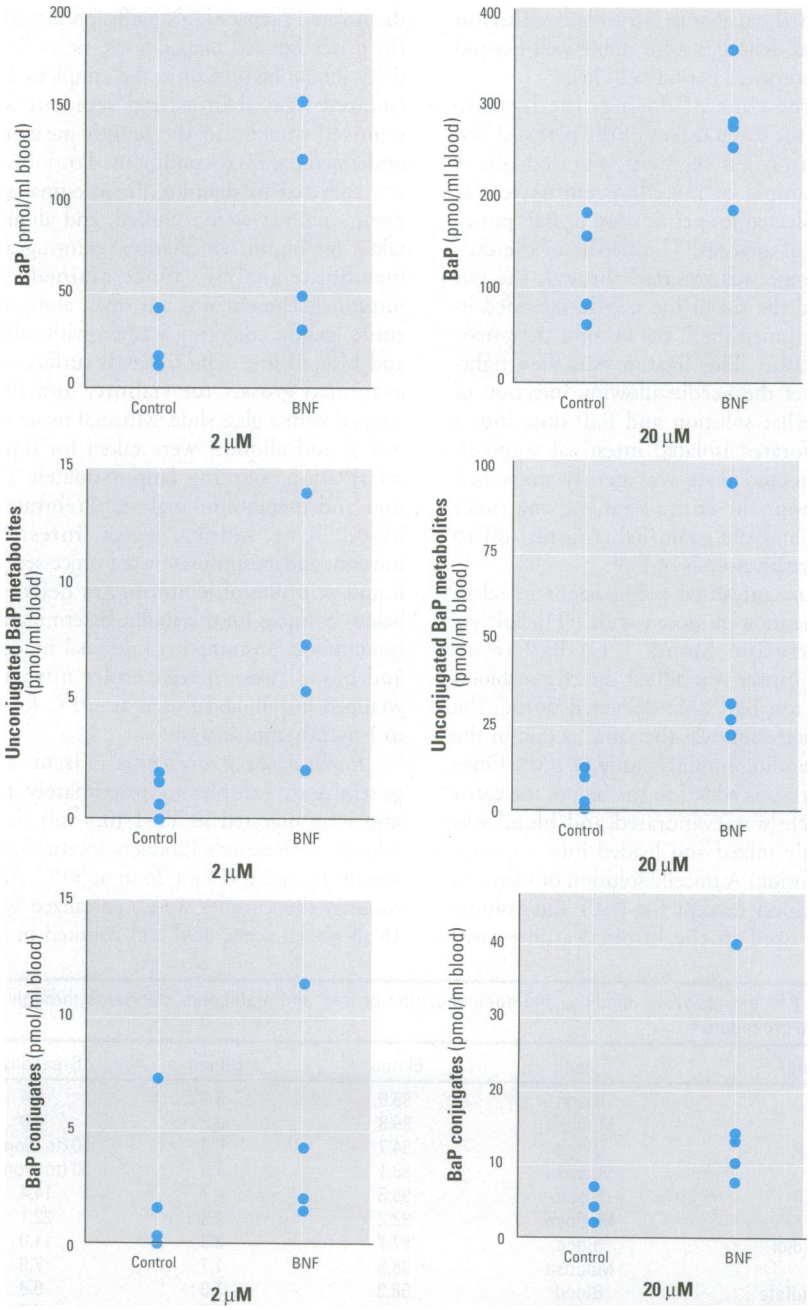

Figure 2. Scatter graphs showing the concentrations of benzo(*a*)pyrene (BaP) and metabolites in the blood from *in situ*-isolated perfused catfish intestine. The four treatment groups were control infused with 2 μM BaP; β-naphthoflavone (BNF)-treated infused with 2 μM BaP; control infused with 20 μM BaP; and BNF-treated infused with 20 μM BaP. Statistical analysis showed significant effects of both BNF treatment and concentration infused for parent BaP and the unconjugated metabolites.

showed that the major metabolites were 3-OH-, 7-OH-, and 9-OH-BaP, BaP-diones, BaP-7,8-dihydrodiol (BaP-7,8-D), and BaP-9,10-dihydrodiol (BaP-9,10-D) (Table 2). 3-OH-BaP accounted for 25–35% of the primary BaP metabolites formed by intestinal microsomes. The apparent K_m for BaP (AHH) in intestinal microsomes from fish fed vehicle-treated chow alone was 1.83 ± 1.06 µM. Intestinal microsomes from catfish treated with BNF demonstrated a significant 5.6-fold increase in the AHH V_{max} value (Table 3). The intestinal microsomes from BNF-treated catfish also had a 6.2-fold higher EROD activity (Table 3). BaP substrate concentrations of 2 µM, close to the control K_m (1.83), and 20 µM (10 K_m) were used for dosage considerations in the *in situ* intestinal preparations.

Total BaP Meq entering the systemic circulation in the *in situ* intestinal preparation varied with dose and with BNF treatment of the fish. A 10-fold increase in BaP dose administered resulted in a significant 3.0–6.3-fold increase in systemically available (blood) BaP Meq (Fig. 2). BNF pretreatment resulted in significant 5.5- and 2.6-fold increases in systemically available BaP Meq for the 2 and 20 µM doses, respectively (Fig. 2). Analysis of radioactivity entering the systemic circulation for each of the *in situ* treatments indicated that the increase in BaP Meq with BNF pretreatment consisted of significant increases in parent BaP and unconjugated metabolites (Fig. 2). Metabolites accounted for 10.2 and 23.1% of the increased BaP Meq in blood of BNF-treated animals for the 2 and 20 µM doses, respectively.

Intestinal mucosa demonstrated significant differences in total BaP Meq with dose. A 10-fold increase in luminal dose resulted in 8.1- and 5.4-fold increases in total mucosal BaP Meq concentrations for control and BNF-treated animals, respectively. Increased BaP Meq with dose was represented by increases in BaP, unconjugated metabolites, and conjugates (Fig. 3). Pretreatment with BNF resulted in a composite 1.8-fold increase in mucosal BaP Meq at the 2 µM dose and a 1.2-fold increase at the 20 µM dose. These changes were also represented by BaP and unconjugated and conjugated metabolites.

A wide variety of primary and secondary metabolites of BaP were formed and transported to the systemic circulation *in situ*. Unconjugated oxidative products included hydroxy-BaP, BaP-diones, BaP-7,8-D, BaP-9,10-D, BaP-7,8,9,10 tetrols, and other minor unconjugated metabolites (Table 4). The *in situ* oxidative profile was similar to those demonstrated in *in vitro* studies, with the exception that hydroxylated metabolites were quantitatively much less important. Conjugates that predictably represented a significant fraction *in situ* consisted primarily of glutathione, sulfate, and glucuronide conjugates. The percentage of total recovered radioactivity as metabolites in the *in situ* preps ranged between 8.3 and 17.3% for blood and 5.3 and 10.2% for the mucosa. For all treatments the mucosa contained much higher absolute BaP and metabolite concentrations than blood (Tables 4 and 5). With the exception of BaP diones at the mucosal 20 µM dose and hydroxy BaP at the blood 20 µM dose, BNF treatment resulted in greater concentrations of metabolites in both the intestinal mucosa and the perfusing blood. This increase was significant for six of the nine reported metabolite classifications in blood and for two of nine in mucosa. When metabolite concentrations were ranked based on their relative abundance among the major metabolites reported, several patterns emerge (greatest abundance = highest rank). Conjugate concentrations were consistently the highest ranked category in both blood and mucosa in both control and BNF-treated animals. BaP diones as a group were the second highest category for all treatments in the blood and the controls at both concentrations for the mucosa. Interestingly, the relative amounts of diones

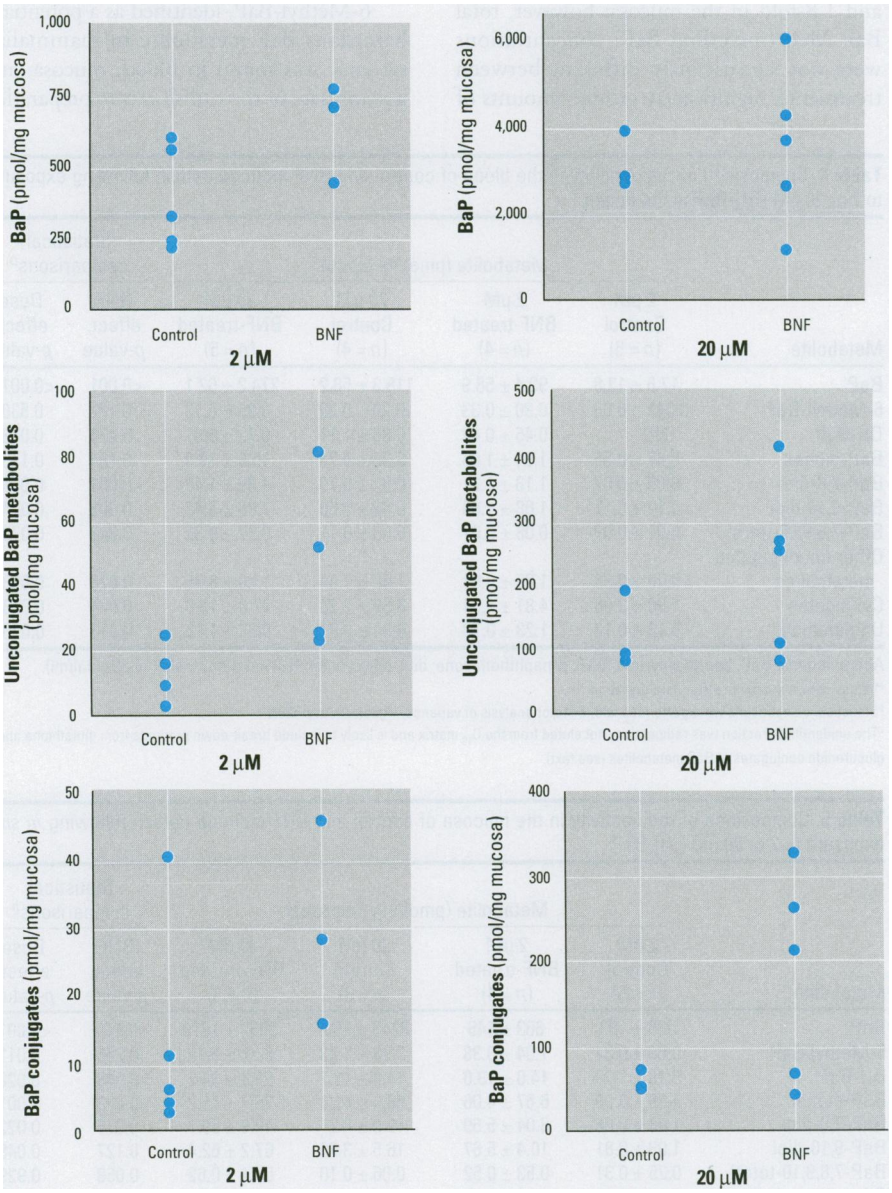

Figure 3. Scatter graphs showing the concentrations of benzo(*a*)pyrene (BaP) and metabolites in the mucosa from *in situ*-isolated perfused catfish intestine. The four treatment groups were control infused with 2 µM BaP; β-naphthoflavone (BNF)-treated infused with 2 µM BaP; control infused with 20 µM BaP; and BNF-treated infused with 20 µM BaP. Statistical analysis showed no significant effects of BNF treatment on the concentrations of BaP or metabolites in mucosa. The concentration of BaP infused significantly affected the concentration of BaP and each group of metabolites in mucosa.

dropped precipitously relative to those of other metabolites in the BNF treatments for both concentrations in mucosa. In terms of ranking among metabolites, relatively more OH-BaP was present in the mucosa than in the blood.

BaP-7,8-D and 9,10-D were evident in blood, mucosa, and postinfusate for both control and BNF treatments at the 2 and 20 μM dosages (Tables 4–6). BaP-7,8-D accounted for 1.8–9.7, 4.2–11.2, and 0.98–2.4% of all metabolites for blood, mucosa, and postinfusate, respectively. In a similar comparison, BaP-9,10-D represented 3.9–13.9, 4.0–16.5, and 0.5–2.6% of all metabolites. Within a category (control or BNF treated), changing the dose from 2 to 20 μM resulted in increased concentrations of BaP-7,8-D and 9,10-D in all samples. BNF treatment resulted in increased BaP-7,8-D and 9,10-D concentrations in blood and mucosa. BaP-7,8-D percentages in BNF-pretreated animals were from 1.3- to 5.4- and 1.5- to 2.7-fold higher than those for controls for blood and mucosa, respectively. Similarly, BNF treatment resulted in BaP-9,10-D percentages that were 3.1- to 3.2 (blood)- and 1.8- to 3.6 (mucosa)-fold higher than those for controls. As a result, BNF treatment elevated the ranking of BaP-9,10-D in blood and both BaP-7,8-D and 9,10-D in mucosa relative to those for other metabolites.

The total BaP Meq, metabolite abundance, and postinfusate volumes recovered from the intestinal lumen following the 1-hr *in situ* exposure are shown in Table 6. Postinfusate volumes recovered were significantly lower with BNF treatment at both the 2 and 20 μM BaP dosages. Average total BaP Meq concentrations in the recovered postinfusate were 744, 1,265, 9,791, and 8,484 pmol for the 2 μM control, 2 μM BNF-treated, 20 μM control, and 20 μM BNF-treated groups, respectively. When the BaP Meq levels were examined on a per-unit basis, the BNF groups consistently demonstrated higher values. BaP Meq of postinfusate, mucosa, and blood on per-unit bases indicate that within a treatment, the BaP Meq of mucosa was 1,855, 667, 1,404, and 546 times higher than that of the postinfusate for the 2 μM control, 2 μM BNF, 20 μM control, and 20 μM BNF treatments, respectively. Similarly, the BaP Meq of mucosa was 20,622, 6,620, 26,427, and 11,902 times higher than that of the blood for the same treatments. For both the mucosa–postinfusate and mucosa–blood comparisons, substantial reductions in the concentration differential within a dose treatment were evident with BNF treatment. Metabolites comprised 12.9, 9.4, 9.5, and 9.0% of the total radioactivity in the postinfusate for the 2 μM control, 2 μM BNF, 20 μM control, and 20 μM BNF treatments, respectively. BNF treatments resulted in elevated average metabolite concentrations for seven metabolite classifications at 2 μM and four metabolite classifications at 20 μM.

The metabolite profiles of radioactivity in postperfusate blood and the intestinal mucosa following [^{3}H]-BaP exposure via infused blood are shown in Table 7. BaP and a wide variety of metabolites were evident in both the blood and mucosa. The major constituent for both the blood and mucosa was the parent BaP. BNF increased the total metabolites twofold in the blood and 1.8-fold in the mucosa; however, total BaP Meq (including BaP) concentrations were not significantly different between treatments. Significantly greater amounts of OH-BaP, BaP-diones, BaP-7,8-D, and BaP-9,10-D were evident in the blood and BaP-7,8-D in the mucosa upon induction than in controls. On an equivalent unit basis, the mucosa had three orders of magnitude higher BaP Meq concentrations than the postinfusate blood. After the BaP parent, the next largest categories in the blood were BaP diones, conjugates, unidentified metabolites, and OH-BaP, respectively, for both control and BNF treatments. The mucosa demonstrated a somewhat different profile from the blood, with the BaP-diones much less prevalent and OH-BaP, BaP-9,10-D, and conjugates more prevalent than the other metabolites.

6-Methyl-BaP, identified as a potentially hazardous BaP metabolite in mammalian systems, was found in blood, mucosa, and postinfusate of the catfish *in situ* preparation

Table 4. Composition of radioactivity in the blood of control and BNF-induced catfish following exposure to 2 or 20 μM [^{3}H]-BaP in the lumen

	Metabolite (pmol/ml blood)[a]				Statistical comparisons[b]	
Metabolite	2 μM Control (n = 5)	2 μM BNF-treated (n = 4)	20 μM Control (n = 4)	20 μM BNF-treated (n = 5)	BNF effect, p-value	Dose effect, p-value
BaP	17.6 ± 13.8	99.4 ± 58.9	118.3 ± 58.2	274.2 ± 62.1	<0.001	<0.001
6-Methyl-BaP	0.041 ± 0.03	0.30 ± 0.33	0.20 ± 0.20	0.25 ± 0.13	0.122	0.530
OH-BaP	ND	0.45 ± 0.42	0.80 ± 1.11	0.79 ± 0.61	0.471	0.073
BaP-diones	0.47 ± 0.55	1.94 ± 1.06	3.38 ± 4.78	15.8 ± 19.0	0.184	0.113
BaP-7,8-diol	0.05 ± 0.07	1.18 ± 1.21	0.62 ± 0.70	4.39 ± 1.48	<0.001	0.005
BaP-9,10-diol	0.12 ± 0.17	1.68 ± 1.68	0.42 ± 0.60	7.05 ± 3.43	0.003	0.024
BaP-7,8,9,10-tetrol	0.01 ± 0.02	0.08 ± 0.07	0.06 ± 0.11	0.37 ± 0.32	0.049	0.079
Other unconjugated metabolites	0.20 ± 0.33	1.65 ± 0.94	1.58 ± 1.94	11.9 ± 8.36	0.026	0.028
Conjugates	1.90 ± 3.06	4.81 ± 4.54	3.69 ± 2.23	17.0 ± 13.0	0.044	0.078
Unidentified[c]	0.13 ± 0.12	1.23 ± 0.78	0.98 ± 0.91	6.63 ± 4.13	0.013	0.020

Abbreviations: BaP, benzo(*a*)pyrene; BNF, β-naphthoflavone; diol, dihydrodiol; ND, not detectable (<0.02 pmol/ml).
[a]Values shown are mean ± standard deviation.
[b]Statistical comparisons were performed with 2-factor analysis of variance; significant at p<0.05.
[c]The unidentified fraction was radioactivity not eluted from the C_{18} matrix and is likely to include break-down products from glutathione and glucuronide conjugates of BaP metabolites (see text).

Table 5. Composition of radioactivity in the mucosa of control and BNF-induced catfish following *in situ* exposure to 2 or 20 μM [^{3}H]-BaP

	Metabolite (pmol/mg mucosa)[a]				Statistical comparisons[b]	
Metabolite	2 μM Control (n = 5)	2 μM BNF-treated (n = 4)	20 μM Control (n = 3)	20 μM BNF-treated (n = 5)	BNF effect, p-value	Dose effect, p-value
BaP	395 ± 183	663 ± 146	3243 ± 650	3562 ± 1816	0.568	<0.001
6-Methyl BaP	0.69 ± 0.32	1.04 ± 0.38	3.40 ± 1.83	6.72 ± 5.19	0.265	0.011
OH-BaP	3.15 ± 1.79	14.0 ± 13.0	14.8 ± 13.7	51.3 ± 34.2	0.048	0.028
BaP-diones	4.28 ± 3.99	6.67 ± 4.06	58.5 ± 41.1	29.7 ± 11.2	0.223	0.001
BaP-7,8-diol	1.08 ± 0.66	8.04 ± 5.59	12.0 ± 8.2	40.9 ± 30.7	0.075	0.022
BaP-9,10-diol	1.03 ± 0.81	10.4 ± 5.67	16.5 ± 3.75	67.2 ± 62.4	0.127	0.048
BaP-7,8,9,10-tetrol	0.25 ± 0.31	0.53 ± 0.52	0.06 ± 0.10	0.74 ± 0.62	0.058	0.929
Other unconjugated metabolites	2.13 ± 2.62	4.43 ± 3.97	14.6 ± 11.2	27.0 ± 12.5	0.128	<0.001
Conjugates	13.3 ± 15.6	26.4 ± 14.2	60.7 ± 12.6	183 ± 122	0.100	0.010
Unidentified	2.27 ± 1.33	11.70 ± 6.41	12.7 ± 8.27	58.9 ± 31.4	0.018	0.008

Abbreviations: BaP, benzo(*a*)pyrene; BNF, β-naphthoflavone; diol, dihydrodiol.
[a]Values shown are mean ± standard deviation.
[b]Statistical comparisons were performed with 2-factor analysis of variance; significant at p<0.05.

for all treatments (Tables 4–7). Compared to other reported metabolites, 6-methyl-BaP was found in small quantities in the blood and mucosa and proportionally larger amounts in the postinfusate. BNF pretreatment did provide a significant increase in 6-methyl-BaP for those fish exposed through the blood (Table 7). Generally, more 6-methyl-BaP was evident with increasing dose for the mucosa and postinfusate.

Discussion

Intestinal CYP1A activity has been examined in several fish species under control and induced conditions. Control AHH activities of intestinal microsomes from vendace, perch, roach (*27*), scup (*28*), toadfish (*15*), and spot (*29*) range from 0.4 to 40 pmol/min/mg protein. EROD activities of 56 and 65 pmol/min/mg have been reported for control spot (*29*) and mummichog (*30*), respectively. Studies examining induction have demonstrated varying responses depending on the species, inducer, and dietary regime (*15,29–31*). Spot exposed to 3-methylcholanthrene (3-MC) (10 mg/kg food for 5 days) had a 17.1-fold increase in AHH activity, whereas the same species administered BaP in the diet (16 mg/kg for 4 days at 2%/day) had an 8.5-fold increase (*29,31*). Toadfish administered BaP in the diet (10 mg/kg food at 5% body weight every third day) experienced a 9.7-fold increase in AHH activity(*15*). AHH activities of induced animals in these studies ranged from 320 to 720 pmol/min/mg protein (*15,29,31*). Similarly, induced EROD activities were 36- and 10-fold higher for spot exposed to 3-MC (*29*) and mummichog exposed to BNF (BNF 250 mg/kg food) (*30*), with activities ranging from 667 to 2,018 pmol/min/mg protein. BNF administration in the present study resulted in an increase in AHH activity from 12.5 to 70.2 pmol/min/mg protein and EROD activity from 1.86 to 11.5 pmol/min/mg. CYP1A intestinal activities of catfish were considerably lower than those of the mummichog (*30*), spot (*29,31*), and toadfish (*15*) for both the respective controls and induced treatments. For those other studies and species for which only controls were examined, the catfish control activities were of comparable magnitude. The higher CYP1A activities in the mummichog, spot, and toadfish relative to that in the catfish may be due to some level of induction in these field-collected fish or to inherently higher basal activities in those species. In addition, for induced animals, BNF has been shown *in vitro* to be an inhibitor for catfish intestinal EROD, AHH, UDP-glucuronosyl-transferase (UGT) and PAPS-sulfotransferase (ST) activities. The concentrations that inhibit 50% (IC_{50}) for EROD, AHH, ST, and UGT were demonstrated to be 0.078 ± 0.022, 2.2 ± 0.09, 48.0 ± 3.0, and 46.9 ± 15.1 μM, respectively (*16*). The catfish intestinal biotransformation enzymes appear to be highly sensitive to inhibition by BNF. This is not true for all fish species, as dietary BNF dosages much higher than those used here have resulted in significant intestinal CYP1A induction (*30*).

Intestinal microsomes from catfish maintained on the control diet produced several major metabolites, including 3-OH-BaP, other benzo-ring phenols, and a variety of dihydrodiols. This group of metabolites was similar to those produced in the liver of other fish species such as sheepshead minnow, killifish (*32*), starry flounder, English sole (*33*), and scup (*34*). Intestinal microsomes of control catfish demonstrated a 18.7-fold lower total BaP metabolite yield than that reported for the liver (*16*). The percentages of individual metabolites, however, were quite similar for 3-OH-BaP (intestine, 38%; liver, 38.5%), 7-OH and 9-OH BaP (intestine, 8.4%; liver, 11.1%), and BaP-7,8-D (intestine, 6.9%; liver, 11.7%). Substantial differences were observed for the diones (intestine 25.4%; liver 10.5%). BaP-7,8-D, a precursor to the major putative carcinogen, was formed to a significantly greater degree as in other fish species and in liver microsomes than by their mammalian counterparts (*35–37*). Although large quantitative differences exist for total BaP metabolite production between hepatic and intestinal microsomes of controls, induction studies utilizing BNF under identical exposure conditions as used presently have demonstrated nearly identical AHH activities for the two organs (*16*). The importance of the intestine relative to the liver in biotransformation of dietary BaP may be linked to the level of dietary induction of the intestine.

Table 6. Composition of radioactivity in intestinal lumen postinfusate medium[a] following exposure of control or BNF-induced catfish for 1 hr to 2 or 20 μM [^{3}H]-BaP

	Metabolite (pmol in postinfusate)[a]				Statistical comparisons[b]	
Metabolite	2 μM Control (n = 5)	2 μM BNF-treated (n = 4)	20 μM Control (n = 4)	20 μM BNF-treated (n =4)	BNF effect, p-value	Dose effect, p-value
Volume after 1 hr (ml)	3.26 ± 1.6	1.13 ± 0.26	4.00 ± 1.80	1.15 ± 0.44	<0.001	0.510
BaP	619 ± 475	1128 ± 243	8724 ± 2338	7571 ± 6570	0.853	<0.001
6-Methyl-BaP	2.51 ± 2.13	4.98 ± 1.90	21.24 ± 13.72	21.69 ± 26.76	0.843	0.027
OH-BaP	1.75 ± 2.36	5.21 ± 4.19	47.0 ± 57.3	22.1 ± 30.9	0.481	0.054
BaP-diones	42.6 ± 47.4	42.1 ± 29.7	549 ± 565	255 ± 125	0.275	0.015
BaP-7,8-diol	0.90 ± 1.10	2.84 ± 1.04	18.84 ± 28.6	10.6 ± 16.2	0.721	0.114
BaP-9,10-diol	0.91 ± 1.85	2.98 ± 1.16	4.55 ± 3.43	11.2 ± 12.4	0.199	0.087
BaP-7,8,9,10-tetrol	0.76 ± 1.28	2.79 ± 2.11	3.67 ± 6.22	8.66 ± 9.62	0.227	0.136
Other unconjugated metabolites	5.59 ± 7.26	9.13 ± 2.90	33.3 ± 26.7	76.7 ± 60.8	0.184	0.013
Conjugates	36.5 ± 24.6	46.7 ± 20.8	235 ± 132	346 ± 158	0.246	<0.001
Unidentified	33.2 ± 54.7	20.1 ± 4.6	154 ± 142	161 ± 87	0.943	0.005

Abbreviations: BaP, benzo(*a*)pyrene; BNF, β-naphthoflavone; diol, dihydrodiol.

[a]The total volume left in the intestine after the perfusion differed significantly between control and BNF treatment groups but was not dependent on initial BaP concentration; initially, 4 ml was infused into each intestinal preparation.

[b]Statistical comparisons were performed with 2-factor analysis of variance; significant at p<0.05.

Table 7. Composition of radioactivity in blood and mucosa of control and BNF-induced catfish following exposure to 2 μM [^{3}H]-BaP in the blood

	Blood (pmol/ml)		Mucosa (pmol/mg)	
Metabolite	Control (n = 7)	BNF-treated (n = 6)	Control (n = 7)	BNF-treated (n = 6)
BaP	123 ± 47	157 ± 38	109 ± 53	114 ± 61
6-methyl-BaP	0.17 ± 0.07	0.35 ± 0.14*	0.11 ± 0.04	0.11 ± 0.05
OH-BaP	0.31 ± 0.23	1.06 ± 0.51*	1.47 ± 1.20	2.38 ± 2.30
BaP-diones	2.97 ± 1.41	6.42 ± 2.53*	0.60 ± 0.45	1.11 ± 0.64
BaP-7,8-diol	0.14 ± 0.14	0.82 ± 0.49*	0.84 ± 0.62	2.03 ± 1.16*
BaP-9,10-diol	0.08 ± 0.08	0.61 ± 0.55*	1.24 ± 0.94	3.13 ± 1.28
BaP-7,8,9,10-tetrol	0.06 ± 0.08	0.08 ± 0.09	0.57 ± 0.58	0.66 ± 0.27
Other unconjugated metabolites	0.31 ± 0.22	0.42 ± 0.25	0.20 ± 0.21	0.27 ± 0.22
Conjugates	1.95 ± 1.36	3.33 ± 1.16	3.56 ± 3.79	6.26 ± 3.95
Unidentified	1.19 ± 0.59	1.66 ± 0.54	1.17 ± 0.42	1.63 ± 0.96

Abbreviations: BaP, benzo(*a*)pyrene; BNF, β-naphthoflavone; diol, dihydrodiol.
*Significantly higher than control, p<0.05.

Within individual treatments of the *in situ* studies, mucosal BaP Meq concentrations were 546–1,855 times higher than those for the postinfusate micelle solution. Similarly, mucosal BaP Meq concentrations were 6,620–26,427 times higher than the blood. The differences for mucosal–blood comparisons were greatest for control treatments at either the 2 or 20 µM dosage regimes (mucosa was 20,622–26,427-fold higher than blood). BaP Meq concentrations in the mucosa, postinfusate, and blood all increased with BNF preexposure. The relative increases, however, were much larger for the blood and postinfusate than for the mucosa. Effectively in face of absolute increases, BNF pretreatment lowered mucosal BaP Meq concentrations relative to either the intestinal lumen or the blood. These findings may be related to several events coinciding with BNF preexposure: 1) an increase in metabolite formation; 2) an apparent reduction in the recoverable micelle volume in the intestinal lumen over the duration of the *in situ* perfusion; and 3) a relatively greater bioavailability of BaP Meq to the systemic circulation.

BNF, in many fish species, is known to be an effective inducer of CYP1A activity (*38–40*) as well as a modest inducer of phase 2 activities such as glucuronosyl transferase (*41*) and glutathione-*S*-transferase (*16,30*). Metabolism may act upon intestinal bioavailability by providing substrates for specific transporters such as the multispecific organic anion transporter (*42,43*) or by metabolizing the lipophilic BaP parent to a metabolite with a polarity more favorably suited for absorption. Previous studies in which 3-OH-BaP was administered into the intestinal lumen of the catfish have shown that primarily the conjugates of 3-OH-BaP were transported from the mucosa to the blood (*44*). Similarly, sulfate and glucuronide conjugates of 9-OH-BaP have been shown to be readily absorbed intact from the intestinal lumen into the systemic circulation of the catfish (*44*). Given the similarity in lipid solubility of 3-OH-BaP and BaP (compared to that in conjugates) and what perhaps may be a ready transport of conjugates, increased metabolism may facilitate movement of lipophilic BaP Meq from the lumen to the circulation. Although the systemic availability of BaP Meq increased 2.6–5.5-fold with BNF pretreatment, the increase in metabolites available to the blood represented only 10.2–23.1% (depending on dose) of the increased Meq entering the systemic circulation. This information suggests that although metabolism was likely to have an effect, it probably is not the sole determining factor in the changes in bioavailability.

Perhaps BNF exposure alters cellular structure and/or integrity thereby affecting bioavailability. BNF has been shown to elicit structural and biochemical changes in fish (*45,46*) as well as to inhibit metabolism (phase 1 and 2) (*16,47*). Brown bullheads exposed to BNF at high concentrations (500 mg/kg diet) in the diet exhibited reduced body weights, physical deformities, and epithelial hyperplasia on body surfaces. Similar effects such as reduced body weights and gill and fin lesions have been observed with other Ah receptor agonists (TCDD, for example) in black bullheads (*48*). On a molecular basis, BNF (100 mg/kg) has been shown to alter hepatic phospholipid metabolism in the rainbow trout, with a 34% increase in microsomal phospholipid content within 24 hr of BNF administration (*46*). The BNF dosages used in the current study were 50 times lower than those exhibiting overt toxicity (*45*), 333 times lower than doses used in lipid studies (*46*), and much lower than those commonly used in hepatic induction studies (*49*). Although histology was not performed and signs of gross toxicity were not evident in *in situ* prepared fish, the selective reduction in perfusate volumes with dietary BNF pretreatment indicates that the catfish gastrointestinal tract was different in some manner because of BNF administration. Reduction in postinfusate volumes over the duration of the perfusion by an unknown mechanism may have increased the BaP concentration gradient from the intestinal lumen to the systemic circulation. This increase in the concentration gradient, a solvent drag phenomenon, or a permeability change may have increased the net influx of BaP Meq.

A third possible explanation for the increase in BaP Meq bioavailability with BNF exposure may relate to the induction of transporter function. Although a specific transporter has not been identified as being a factor in these studies, it is known that BNF induces the function of at least one transporter in the intestine of catfish (*50*). This transporter, P-glycoprotein, is known to transport BaP in mammalian liver cells (*51*). It is not known if metabolites of BaP are similarly transported.

When *in situ* intestinal study comparisons are made between metabolism of catfish in the current study and toadfish fed BaP (a weaker inducer than BNF) at 10 mg/kg food at 5% of body weight every 3 days, we find that approximately 8.3–17.3% of BaP in the blood was metabolized in the catfish preparation compared to 62–90% for the toadfish (*15*). *In vitro* AHH activities for pretreated control and induced toadfish had values of 33 and 320 pmol/min/mg microsomal protein, respectively. These values are nearly threefold higher than controls and 4.6-fold higher than induced activities, as demonstrated for catfish in the current study. Such apparent differences in levels of biotransformation may account for the large differences in amounts of metabolites produced in the *in situ* intestinal preparation between the two studies. In addition to enzymatic activities, several experimental differences may contribute to the contrasting *in situ* studies. These differences include species (catfish vs. toadfish), species source (hatchery vs. field), inducer selection (BNF vs. BaP), length of perfused intestinal segment (isolated segment vs. whole intestine), circulatory perfusion method (closed vs. open), BaP carrier vehicle (micelle vs. oil), and the method of metabolite analysis (HPLC vs. differential extraction). *In vivo* studies with rats (*52*) and fish (*53*) also indicate extensive metabolism of dietary BaP by the intestine. Further study is necessary to determine if differences are associated with the species or the experimental protocols.

In the *in situ* preparations, high concentrations of BaP-7,8 and 9,10-D were found in the intestinal mucosa relative to those in the blood and micelle postinfusate. These dihydrodiols appear to arise from the relatively robust activity of catfish mucosal epoxide hydrolase on the respective BaP-oxides (*16*). It is likely that the higher BaP-7,8 and 9,10-D concentrations present in the intestinal mucosa are primarily a result of buildup at the site of production, as orally administered BaP-7,8-D has been shown to be readily transferred from the fish intestine (*54*). Quantitatively, the proportions and absolute concentrations of BaP-7,8 and 9,10-D were greater for BNF-pretreated animals. BNF pretreatment resulted in 1.3–5.4-fold and 1.5–2.7-fold increases in BaP-7,8-D percentages for blood and mucosa, respectively. Comparisons for BaP-9,10-D demonstrated similar differences, with blood and mucosa attaining 3.1–3.2-fold and 1.8–3.6-fold higher percentages with BNF treatment. Although direct intestinal or *in situ* comparisons are unavailable, incubation of BaP with hepatic microsomes from induced and uninduced scup (*55*), southern flounder (*37*), starry flounder, and English sole (*33*) resulted in elevated dihydrodiol concentrations and either small increases, no change, or decreases in the percentage of total metabolites as dihydrodiols following *in vivo* induction. In most fish studies, induction with CYP1A inducers results in increased concentrations of dihydrodiols as well as other metabolites. Studies with catfish hepatic microsomes, as well as studies here with the intestine, indicate not only indiscriminate increases in metabolites but some selectivity in BaP-7,8-D and 9,10-D formation upon induction.

Proportional increases in the dihydrodiol contribution with induction have been demonstrated in a variety of mammalian studies and are due to induction of epoxide hydrolase as well as CYP1A (*56*). In the catfish intestine the elevated dihydrodiol concentrations with BNF pretreatment appear to be due to increased CYP1A activities in face of high but noninduced levels of epoxide hydrolase activity (*16*). Importantly, the catfish intestine responds to dietary BNF by producing greater proportions of potentially hazardous BaP metabolites that may be transported by the systemic circulation to distant sites. Classic toxicokinetic studies with intravenous BaP and BaP-7,8-D in the winter flounder indicate higher volumes of distribution (Vd) and clearance rates with BaP-7,8-D (*54*). Clearly these features offer contrasting risks because the increased polarity of BaP-7,8-D facilitates elimination, whereas a larger volume of distribution poses greater access of a more proximate carcinogen (BaP-7,8-D) to susceptable macromolecules.

6-Methyl-BaP and 6-OH-methyl-BaP (data not included) were shown to be formed in the catfish *in situ* intestinal preparation. To the authors' knowledge this is the first time these metabolites have been tentatively identified in a fish species. 6-Methyl-BaP has been demonstrated in mammals to arise *in vivo* from the methylation of BaP by methyl transferase (*57*), whereas 6-OH-methyl-BaP results from a P-450-mediated hydroxylation of the methylated metabolite. 6-Methyl-BaP and 6-OH-methyl-BaP as well as the sulfate, phosphate, and acetate esters of 6-OH-methyl-BaP, when given to animals, have been shown to form protein and DNA adducts through the 6-methylene group (*58*). These adducts may arise from an S_N2 (substitution nucleophilic biomolecular) reaction of the ester of 6-OH-methyl-BaP with cellular nucleophiles or by the sulfation of 6-OH-methyl-BaP and loss of the sulfate anion possibly to form a reactive carbocation (*58,59*). Further investigation will be necessary before the importance of these potential pathways of BaP activation are known for the fish intestine.

Diones were major contributors to the total metabolites in the *in situ* intestinal preparation as they were for the microsomal positional metabolism. Intestinal mucosa contained much higher concentrations of diones on a per-unit basis than either the blood or postinfusate. In general, the amount of diones increased from the 2 µM to the 20 µM dose for both control and BNF comparisons. BNF pretreatment resulted in increased dione concentrations for both the 2 µM and 20 µM concentrations of the blood and the 2 µM concentration in the mucosa. For the 20 µM treatment, dione concentrations in mucosa demonstrated a large drop with BNF pretreatment. Diones constituted a lower percentage of the total metabolites for BNF treatments than in controls for mucosa and marginally lower in the blood. This response is in contrast to the relative increases in percentages of BaP-7,8 and BaP 9,10-D on BNF pretreatment. A likely explanation for these findings is that induction of CYP1A by BNF favors formation of benzo-ring arene oxides, the precursors to benzo-ring dihydrodiols.

When BaP was administered into the intestinal lumen of the *in situ* preparations, between 33.9 and 60.1% (depending on treatment) of the metabolites in the postinfusate were diones. This compares to 7.3–32.4% for the mucosa and 16.0–31.4% for the blood across identical treatments. For studies where BaP was administered through the blood, diones accounted for 49.6 and 49.0% of the blood metabolites for the 2 µM control and BNF treatments, respectively. The mucosa in these same treatments contained between 6.9 and 7.5% of total metabolites as diones. Although the mucosa contained the highest absolute dione content, it also accounted for the greatest BaP Meq concentrations and total net metabolism. Conversely, the postinfusate and blood, although not producing metabolites to the same degree as mucosa, were represented to a much greater degree by dione formation. There are interesting corollaries of these observations to the situation in many invertebrates in which P450 metabolism is low and dione formation is of greater importance (*60*). Although comparisons with intestinal or *in situ* preparations are not available, the percent of metabolites as diones has been reported for hepatic microsomes from a variety of fish species (*6*). Of the studies examined, the percent of diones formed by fish ranged from 3.8 to 28%, with a median value of approximately 13%. The percent of metabolites as diones reported here for the *in situ* intestinal preparation generally fell into this range except for the high values for the postinfusate across all treatments and the postperfusate blood (control and BNF pretreated) of the blood exposure studies. Large-scale air oxidation of the BaP appears to be an unlikely explanation for the high levels of diones in these samples because preinfusate handled in an identical fashion but without exposure to tissues did not exhibit significant dione production. Pathways of xenobiotic oxidation that characteristically form BaP diones have been identified with the vasculature and hematin of mammals (*61*). Dione formation in mammals is thought to occur by the intermediacy of a BaP cation radical (*62*) and 6-OH-BaP (*63,64*) through a one-electron oxidation mechanism. This oxidation may occur with BaP acting as a reducing cosubstrate for the oxidation of arachidonic acid by prostaglandin H synthase (PHS) or by reactions catalyzed by heme containing proteins and lipid hydroperoxides. Tissues with low monooxygenase activity and high PHS concentrations such as the alimentary tract and vasculature are favorable sites for this oxidation route (*61*). Concentrations of PHS in fish tissues are unknown; however, P450 activities generally are lower in the vascular (*65–67*) and intestinal (*16*) compartments than in the liver, especially in uninduced animals. Predictably, an attempt with liver microsomes to detect the 6-oxy-BaP free radical in fish was largely unsuccessful (*6*). Non-P450-dependent metabolic routes and transport differences in the intestine and the vasculature are plausible explanations for the prominent role of diones in these select studies and samples.

Conjugates represented a major component in the blood and intestinal mucosa, accounting for between 26.5 and 65.0% and 31.4 and 51.3% of the recoverable metabolites, respectively. Blood and mucosa of controls demonstrated greater composite conjugate concentrations than the unconjugated metabolites at the 2 µM dose, whereas at the 20 µM dose the unconjugated metabolites attained 1.9–2.0-fold higher concentrations. BNF treatment, while increasing absolute concentrations of all metabolites, appeared to negate these findings, as the unconjugated metabolites predominated at both the 2 and 20 µM concentrations. Both dose and BNF pretreatment appeared to facilitate in absolute terms the increased production of intestinally derived metabolites. Conjugate concentrations, although sensitive to these mechanisms, demonstrated incongruencies with the larger response of the unconjugated metabolites. Relative limitations in phase 2 metabolism in face of increased concentrations of phase 1 metabolites may account for the observed findings. This concept is supported by previous BNF induction studies with catfish intestine that have demonstrated little induction of glutathione-*S*-transferase and no induction of UGT or ST (*16*). In addition, BNF treatment also increases CYP1A production of BaP-7,8-oxide and BaP-9,10-oxide, which in turn leads to increased BaP-7,8-D and 9,10-D formation, as well as 7- and 9-hydroxy BaP (Fig. 3). The resulting BaP-dihydrodiols are poorer substrates for glucuronidation than BaP-phenols (*68*), which leads to a buildup of unconjugated dihydrodiols relative to hydroxy-BaP. Strict dose effects indicate that such limitations may also be related to

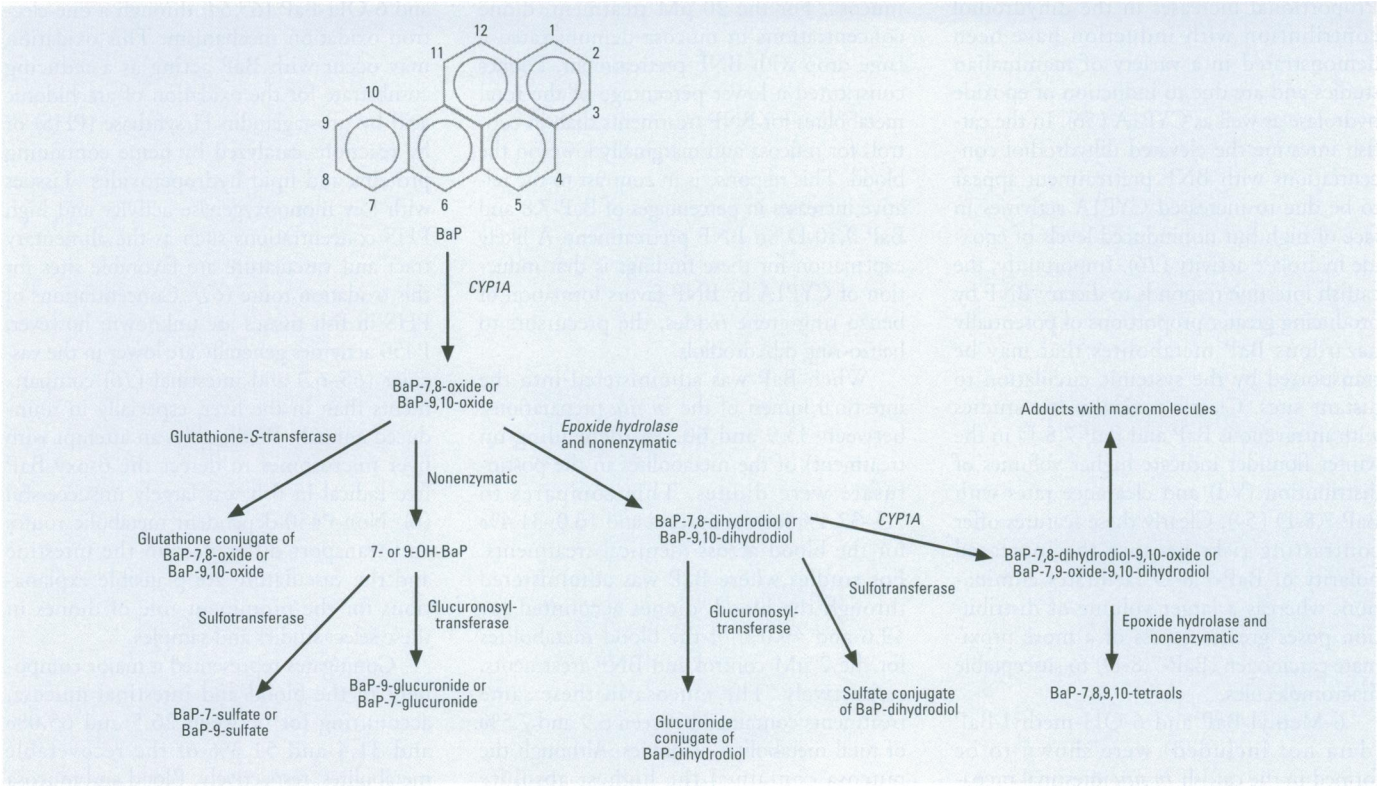

Figure 4. Metabolic cascade for benzo(*a*)pyrene (BaP) via the cytochrome P4501A (CYP1A) products BaP-7,8-oxide and BaP-9,10-oxide.

K_m/V_{max} as well as availability considerations. Intestinal mucosal homogenates from flounder administered dietary BNF when incubated with BaP at 0.5 and 5.0 μM exhibited greater amounts of BaP conjugates, whereas at 50 and 250 μM, conjugates fell relative to the polar metabolites and unmetabolized BaP (*17*). A similar dose dependence appears to exist here for the *in situ* intestinal metabolism of BaP.

The water-soluble fractions and HPLC peaks eluting before 10 min in the present study are likely to include a mixture of sulfate, glucuronide, and glutathione conjugates of the oxidative metabolites of BaP (Fig. 4). The large number of potential conjugates from BaP-phenols, BaP-dihydrodiols, and BaP-oxides, in light of their overlapping physicochemical properties, made isolation of individual polar conjugates difficult based on retention characteristics. Incubation of radioactivity eluted from the C_{18} matrix beads by water and methanol with β-glucuronidase or sulfatase suggested that up to 20% of the conjugates found were a mixture of glucuronides and sulfates. More than 80% of the conjugates were not hydrolyzed by sulfatase or β-glucuronidase, suggesting that these were glutathione conjugates and further metabolites of glutathione conjugates. Others have shown that glutathione conjugates were the major metabolites of orally administered BaP (0.1–2 mg/kg) excreted *in vivo* in bile of several fish species (*6,69*). In addition to the water and methanol fractions eluted from the C_{18} matrix beads, there is evidence that a portion of the unidentified radioactivity not eluted from the C_{18} beads included breakdown products of glucuronide and glutathione conjugates [Table 1; Tong and James, unpublished observations; (*70*)].

Intestinal microflora are thought to be minimal contributors to the observed findings. There is no evidence that these organisms contain the P450s or conjugative enzymes involved in BaP oxidation or conjugation [Fig. 4; (*71–73*)]. Microflora, however, do contain reductive and hydrolytic enzymes. Degradative reactions such as dehydroxylation and hydrolysis of glucuronides may decrease postinfusate metabolite concentrations. This effect is thought to be minimized because fasting as well as clearing fecal material would be expected to reduce bacterial numbers and their metabolic contribution (*74*).

A considerable degree of variation was evident in all aspects of these studies. Fish are inherently variable relative to a number of key biotransformation enzymes (*75*). Intestinal studies pose other challenges. Catfish because of their poikilothermic nature and feeding behavior show variations in food consumption and hence in *in vivo* intestinal induction. This is problematic because healthwise they do not tolerate long-term repetitive handling as in gavage feeding. In addition, variable boundaries imposed by the circulation in perfusion studies, the somewhat variable length of the perfusion segment because of the state of relaxation and contraction of the *in situ* intestine, the wide scope of available biotransformation pathways, and the dynamic nature (turnover) of the mucosal cells containing uptake and biotransformational sites all contribute to variability.

In summary, the systemic bioavailability of BaP molar equivalents from the intestine was significantly greater with higher BaP dosages and BNF pretreatment. BNF generally increased metabolite production; however, increases in bioavailability could not be accounted for solely by changes in metabolism. Intestinal formation and systemic uptake of potentially hazardous metabolites and detoxification products of BaP in the catfish intestine appear to be related to dose and BNF pretreatment through an interplay of primary and secondary biotransformation pathways. Conjugates were proportionally favored under low-dose uninduced conditions, whereas benzo-ring diols were proportionally more important upon BNF preexposure. Clearly the interplay of dose, exogenous agents, and intestinal function are important factors in the uptake, disposition, and risk associated with dietary BaP.

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